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ABSTRACT

Pre-menopausal patients whose breast cancers are, in general, estrogen receptor (ER) negative and biologically more aggressive, are particularly in need of novel therapeutic interventions. A breast cancer vaccine to prevent relapse after conventional treatment would be of enormous clinical value. Several cancer vaccine studies have demonstrated that breast cancer patients can develop tumor antigen specific immune responses after a vaccine is given. Most of these approaches target a single immunogenic protein or antigen thus limiting the vaccine to those patients whose tumor expresses that antigen. We propose the development of a multi-antigen vaccine that could potentially benefit any ER negative breast cancer patient; the category of patient at highest risk of relapse. Several high throughput antigen discovery tools have been developed that have greatly helped the identification of immunogenic proteins in breast cancer patients. One such technique, SEREX (serological analysis of cDNA expression libraries) identifies tumor antigens based on spontaneous antibody immunity that can occur in breast cancer patients. To date, over 2,000 tumor antigens have been identified from a variety of cancers using SEREX. In fact, so many tumor antigens have been identified a major question facing tumor immunologists today is- which antigen will induce anti-tumor immunity? Investigators evaluating immunity to leukemia have recently utilized patients who develop an anti-tumor response after immunotherapy to identify true "tumor rejection" antigens, that is, those immunogenic proteins which, if targeted, would induce an anti-tumor response. This work was possible because one of the treatments of relapsed leukemia is immunotherapy using donor lymphocyte infusions. The investigators could identify which immunogenic proteins were associated with the development of a remission after immunotherapy. Unfortunately, immunotherapy has not yet advanced to a stage in the treatment of breast cancer where we can reproducibly induce tumor regression. We have recently determined that the neu transgenic (neu-tg) mouse can serve as an excellent model for identifying human breast cancer antigens. These mice were genetically engineered to develop breast cancer that is almost identical to human breast cancer. The breast cancers that occur in these mice are ER negative and drug resistant mimicking pre-menopausal breast cancer in women. Preliminary experiments have shown that neu-tg FVB/N mice may share the same pool of tumor antigens with breast cancer patients. Our goal in this proposal is to identify antigens that are associated with tumor rejection. Whereas this study would not be possible in humans, we have recently established a tumor rejection model by implanting the mouse tumors derived from neu-tg mice into the parental FVB/N mouse, who are identical in every way except were NOT engineered to develop breast cancer. In our model, none of the parental FVB/N mice develop tumor while all of the neu-tg mice that received tumor implantation succumb to their disease despite having endogenous immunity to some proteins expressed by the tumor. Interestingly, the tumor rejection that occurs is not solely mediated by immunity to neu- the major cause of the cancer (similar to human HER-2/neu). We proposed to use subtractive SEREX, a method established in the laboratory to screen for antigens that are specifically induced by tumor rejection. The tumor antigens that have immunogenic human homologues will be further studied by using them to vaccinate the neu-tg mice to see if such a vaccine will prevent the cancers. The human homologues of these proteins, identified as described in this proposal, will be the basis for a multi-antigen vaccine to prevent breast cancer relapse in pre-menopausal patients with ER negative breast cancer.

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Introduction

The purpose of this study is to identify tumor rejection antigens using mouse tumor rejection models. Although a number of tumor antigens have been identified in cancer patients, it remains a challenge to identify therapeutically relevant tumor rejection antigens. Previous studies in our lab have demonstrated that the tumor antigen repertoire in tumor-bearing neu transgenic mice has great similarity to the antigen repertoire in breast cancer patients. Immune response-mediated tumor regression, although difficult to achieve in cancer patients, have been reported in mice. The goal of the proposed study is to identify tumor rejection antigens using mouse tumor rejection models. The study has three specific aims: (1) to determine the antigen repertoire induced by tumor rejection in FVB/N mice; (2) to identify the human homologues of the candidate rejection antigens and determine their immunogenicity; and, (3) to examine the *in vivo* tumor protection effect of vaccination with plasmids encoding tumor rejection antigens in neu-tg FVB/N mice. In the first funding year of this grant, we successfully identified the antigen repertoire induced by tumor rejection in FVB/N mice. The human homologues of the candidate rejection antigens have been determined by database mining. In the second funding year period, we investigated the potential tumor protection effect of the identified novel antigens using in vivo plasmid DNA vaccination experiment and prioritized the antigens to be tested in human. We are well posed to examine the immunogenicity of the most promising candidate antigens in cancer patients in the last year of the funding period.

Key Research Accomplishments

Specific Aim 1: to determine the antigen repertoire induced by tumor rejection in FVB/N mice.
This was completed in the first funding year (2006-2007). In brief, we have established two mice tumor rejection models. SEREX screening by comparing the serum antibody repertoire prior to and post to tumor rejection identified a panel of 10 tumor rejection antigens, including FxyD3, Cep290, Ctnna1, Tln1, Hsp40, GPIap1, Tnaaip3, Hnrpl1, Tmem57, and Mtv1. This antigen panel is totally different from the antigen panel we previously identified in tumor-bearing mice, indicating that the antigens mediating the tumor destructive immunity may be distinct from the antigens that induce immune tolerance to tumor (Table 1).

Specific Aim 2: to identify the human homologues of the candidate rejection antigens and determine their immunogenicity.
By searching published literature and database mining, we have found that six out of the ten tumor rejection antigens have previously been shown to have immunogenic human homologues, including Ctnna1, Hsp40, Cep290, Tln1, Hnrpl1, and Tnaaip3.

The immunogenicity of the tumor rejection antigens will be further examined using serum and PBMC specimens from breast cancer patients. However, we will only test the antigens that show tumor protective effect in animal testing. Therefore we have hold on the testing in human until we finish the in vivo vaccination experiment in mice (Aim 3). This will be the major task in year 3.

Table 1. Comparison of tumor antigens identified in tumor-bearing mice and tumor-rejecting mice

Antigens in Tumor-rejecting Mice	Gene Symbol	Antigens in Tumor-bearing Mice	Gene Symbol
Mouse mammary tumor virus	Mtv1	Cytokeratin 8	Krt2-8
Catenin (cadherin-associated protein) alpha 1	Ctnna1	Glutamyl-prolyl-tRNA synthetase	Eprs
Heat shock protein 40	Hsp40	Compliment C3	C3
Transmembrane protein 57	Tmem57	Galectin 8	Lgals8
Centrosomal protein 290 kDa	Cep290	Serine-threonine rich protein kinase 1	Srpk1
FXYD domain containing ion transport regulator 3	Fxyd3	RAB3A interacting protein	Rab3ip
Talin 1	Tln1	Rho-associated coiled-coil containing protein kinase 1	Rock1
Heterogenous nuclear ribonuclear protein L-like	Hnrpl	Schistosoma mansoni adult worm antigen preparation	Swap70
GPI-anchored membrane protein 1	GPIap1	Rho/rac guanine nucleotide exchange factor 2	Arhgef2
TNF alpha-induced protein 3	Tnfaip3	Gelsolin	Gsn
		Nuclease sensitive element binding protein 1 (Y Box binding protein 1)	Nsep1 (YB1)
		Ubiquitin specific protease 7	Usp7
		Leucine zipper transcription factor like 1	Lztfl1
		Exportin 5	Xpo5
		Matrin 3	Matr3

Specific Aim 3: to examine the *in vivo* tumor protection effect of vaccination with plasmids encoding tumor rejection antigens in neu-tg FVB/N mice.
Multiple vaccination experiments were carried out in the last year to prioritize the identified tumor rejection antigens based on their *in vivo* tumor protective effects. Female neu transgenic mice received plasmid DNA encoding the different tumor antigens. Plasmid DNA encoding the empty vector (pBK-CMV) was included as negative control. Plasmid DNA encoding the intracellular domain of human HER2 (hICD), was included as positive control. Mice were given three vaccines intradermally (50ug plasmid DNA per vaccine), two weeks apart. Tumor challenge using a syngeneic tumor cell line was give at two weeks after the third vaccine. The tumor growth was measured twice a week using venier calipers. Results from multiple vaccination experiments suggests the following important findings-

1. Vaccination using tumor rejection antigens but not other tumor antigens has tumor protection effect. As shown in Fig. 1, vaccination using Mtv1, FxyD3, and Cep290 had tumor protection effect. In contrast, vaccination targeting the antigens we previously identified from tumor-bearing mice, such as Swap70 and Rock1, did not have tumor protection effect (Fig. 2). To further confirm the observation that antigens from tumor-rejecting mice but not tumor-bearing mice had tumor protection effect, we tested the 2 groups of antigens side-by-side in one experiment. As shown in Figure 3, the tumor growth curves separated into two groups. The first group of mice had the same tumor growth rate as the mice received empty vector (negative control). These included mice received Rock1, Gsn, Swap70, and Eprs, all of which were identified from tumor-

bearing mice. The second group of mice had the same growth rate as the mice received hICD (positive control). These include mice received FxyD3, Tmem57, Mtv1, and Cep290, all of which were identified from tumor rejection mice. This experiment confirmed previous findings that vaccination targeting antigens identified from tumor rejection mice but not antigens identified from tumor-bearing mice had tumor protection effect.
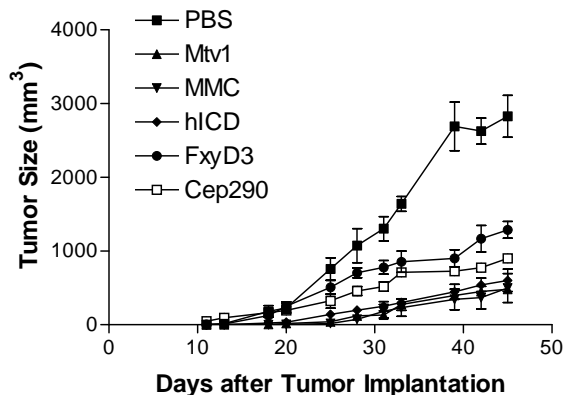

Figure 1. Vaccination targeting tumor rejection antigens, Mtv1, FxyD3, or Cep290, resulted in tumor protection. Mice (3 per goup) were vaccinated with plasmid DNA encoding Mtv1, FxyD3, Cep290, or hICD, or irradiated whole tumor cells at day -42, -28, and -14. Live MMC cells were given subcutaneously on day 0.
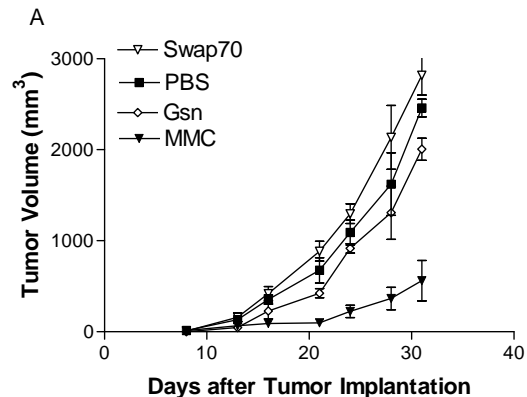
Figure 2. Vaccination targeting tumor antigens identified from tumor-bearing mice, Swap70 and Gsn, did not have tumor protection effect. Mice (3 per goup) were vaccinated with plasmid DNA encoding Swap70 or Gsn, or irradiated whole tumor cells at day -42, -28, and -14. Live MMC cells were given subcutaneously on day 0.
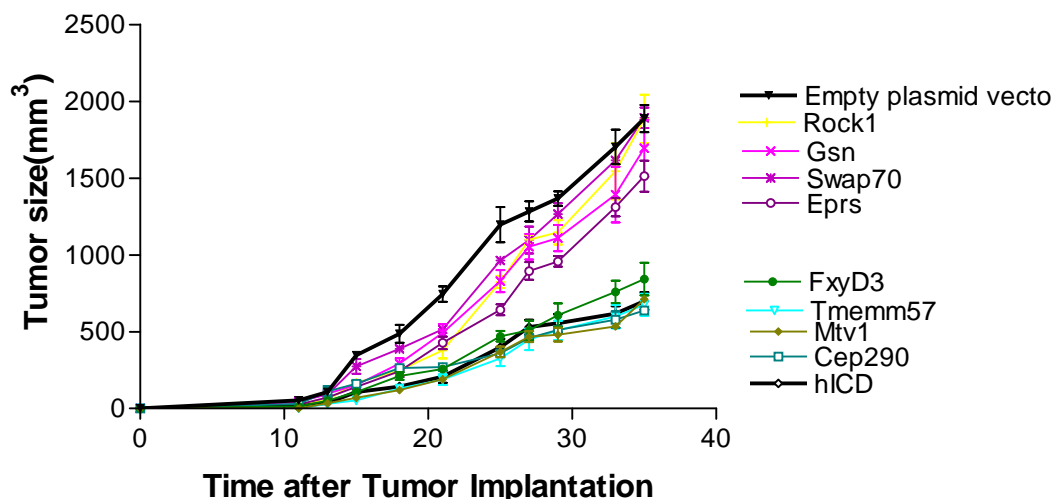

Figure 3. Vaccination targeting antigens identified from tumor-rejecting mice but not tumor-bearing mice had tumor protection effect. Four tumor rejection antigens and 4 non-tumor rejection antigens were examined side-by-side in one experiment. Mice were vaccinated with plasmid DNA encoding each antigen on days -42, -28, and -14. Tumor challenge was given on day 0.

For the best candidate antigens (FxyD3, Cep290, and Mtv1), we also started testing their tumor protection effect in spontaneous tumor models. Female neu-tg mice (6-8 weeks old) first received 3 vaccinations, 2 weeks apart. Since spontaneous tumors don't develop in these mice until they are 5-7 months old. The mice will receive 3 boost vaccines once a month after the initial 3 doses of vaccine. Tumor development will be followed until the mice are 1 year old. The result from this experiment is still pending.

2. Multiple antigen vaccine is superior to single antigen vaccines. The goal of the proposed study is to identify novel tumor rejection antigens that can potentially be used together with currently available vaccine (hICD) for breast cancer. Therefore, we examined the potential synergistic effect of combining newly identified antigen vaccine together with hICD vaccine, which was known to have tumor protection effect in these mice. As shown in Figure 4, combination of antigens worked better than single antigen. The tumor protection effect of 2 antigens (Mtv1+FxyD3) was better than either agent alone. The combination of three antigens (Mtv1+FxyD3+hICD) provided the most tumor protection effect. In the next funding year, we will test this best combination of vaccine in spontaneous tumor models.
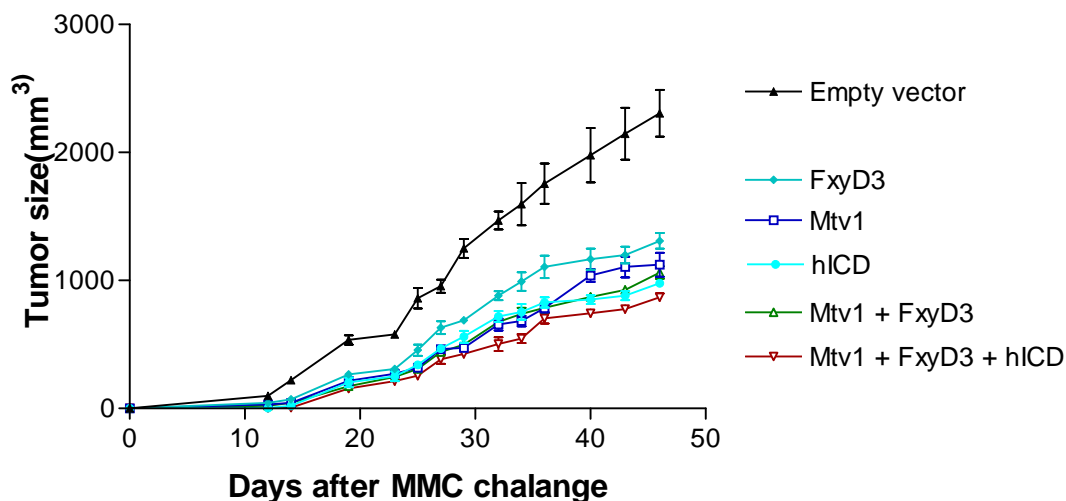

Figure 4. Multi-antigen vaccines worked better than single antigen vaccines. Six-eight weeks old female neu-tg mice were vaccinated with either a single plasmid DNA (FxyD3, Mtv1, hICD, or empty vector) or combination of plasmid DNAs (Mtv1+FxyD3 or Mtv1+FxyD3+hICD) on days -42, -28, and -14. One million live MMC cells were given subcutaneously on day 0.

Reportable Outcomes

Publication:
1. Lu H, Knutson KL, Gad E, Disis ML. The tumor antigen repertoire identified in tumor-bearing Neu transgenic mice predicts human tumor antigens. Cancer Res 2006; 66: 9754-61.

Presentations:
1. Lu H, Gad E, Chang A, Seymour K, and Disis ML. The identification of tumor rejection antigens in murine models that are associated with human homologues. Poster presentation at AACR, 2006

2. Lu H, Chang A, Larson E, Gad E, and Disis ML. Identification of an immunological signature of tumor rejection in the neu transgenic mouse. Oral presentation at AACR, 2007

3. Lu H, Chang A, Gad E, Larson E, Park E, La S, Disis ML. Vaccination targeting antigens identified in tumor rejection mice but not antigens identified in tumor bearing mice has tumor protective effect. Poster presentation at DOD Era of Hope meeting, 2008

Conclusion and Future Directions

Studies during the first 2 funding years have shown that tumor rejection antigens can be identified from tumor rejection mice. The panel of antigens identified from tumor rejection mice is totally difference from the panel of antigens identified from tumor bearing mice. In the past year, we focused on using in vivo vaccination experiments to prioritize the newly identified antigens. We found that the antigens from tumor rejecting mice are superior to the antigens identified in tumor bearing mice in preventing tumor development. Furthermore, we found that a multi-antigen vaccine composed of 3 antigens worked better than each antigen alone. In addition to testing the new vaccines using a tumor implant model, we have started testing their efficacy in spontaneous tumor model with pending results. For the next funding year, we will finish the vaccination experiment in spontaneous tumor model and also move on to evaluate the immunogenicity of the best candidate antigens in human setting. Our group has banked serum and PBMC specimens from breast cancer patients and normal donors, which allow us to test the potential serum antibody and T cell response to these antigens in cancer patients. The results from these studies will be used to prepare an IND application for Phase I clinical trial testing the new antigens in cancer patients.